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13. ABSTRACT ***(Maximum 200 Words)***

The effects of ginseng on the prevention and treatment of breast cancer has not been studied. This research project was designed to examine the effects of American ginseng on breast cancer using well-established in vitro and in vivo experimental models. It is our hypothesis that ginseng, and its ginsenosides in particular, would inhibit the proliferation and growth of human breast cancer cells. Our results have shown that an extract of American ginseng inhibited MCF-7 and MDA-MB-231 cell proliferation in a dose-dependent manner. Furthermore, ginseng extract in the drinking water of female nude mice significantly decreased human breast cancer tumor growth. We have identified several different ginsenosides in ginseng extract and determined that only ginsenosides Rc and Rh2 potently inhibited breast cancer cell proliferation *in vitro*. These findings are the first to suggest that these ginsenosides may be responsible for the anti-proliferative actions of ginseng extract on human breast cancer cell proliferation. More recent data suggested that ginsenoside Rc may inhibit breast cancer cell cycle progression, whereas ginsenoside Rh2, especially at higher doses, is cytotoxic. Thus, different ginsenosides may have very different mechanisms of action.

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Introduction

The ingestion of Asian ginseng or ginseng root components has been reported to reduce the risk of human cancer and has been shown to decrease the rate of occurrence and proliferation of cancers in experimental animals. However, the effects of ginseng on the prevention and treatment of breast cancer has not been studied. This research project was designed to examine the effects of American ginseng (*Panax quinquefolium*) on breast cancer by studying the effects of novel ginseng preparations 1) on well-established human breast cancer cell models *in vitro*, 2) on human mammary tumor xenografts in nude mice *in vivo*, and 3) on carcinogen-induced mammary tumor development in female rats. Our hypothesis stated that specific ginseng components would inhibit the proliferation and growth of human breast cancer cells and would reduce the incidence of mammary tumor development in experimental animals. As breast cancer is one of the most prevalent and deadly diseases afflicting women today, a means of preventing and/or treating breast cancer through dietary supplements would have a significant impact on women's health.

Body

This is the third and final annual report of the grant "American Ginseng in the Prevention and Treatment of Human Breast Cancer." In the Statement of Work, three tasks were described as follows:

Task 1. Testing effects of American ginseng extract on human breast cancer tumor growth in nude mice and on mammary tumor formation in carcinogen-treated female rats.

a. Prepare water extract of ginseng for HPLC analysis (month 1)
b. Order mice and treat water with ginseng extract (months 1-6)
c. Grow MCF-7 and MDA-MB-231 cells for inoculation (months 1-6)
d. Inoculate mice and monitor tumor growth (months 1-8)
e. Order rats and treat water with ginseng extract (months 7-12)
f. Treat rats with NMU and monitor tumor formation (months 8-18)

Task 2. Testing effects of ginsenosides on MCF-7 and MDA-MB-231 cell proliferation *in vitro*.

a. Grow and plate MCF-7 and MDA-MB-231 cells for inoculation (months 6-18)
b. Treat cells with ginsenosides and measure proliferation (months 6-18)

Task 3. Testing effects of ginsenosides on human breast cancer tumor growth in nude mice and on mammary tumor formation in carcinogen-treated female rats.

a. Order mice and treat water with ginsenoside combinations (months 19-28)
b. Grow MCF-7 and MDA-MB-231 cells for inoculation (months 20-24)
c. Inoculate mice and monitor tumor growth (months 21-28)
d. Order rats and treat water with ginsenosides (months 26-35)
e. Treat rats with NMU and monitor tumor formation (months 27-35)

Per Task 1, a water extract of ginseng was prepared as described in Methods section of grant (Task 1a). A sample of lyophilized extract was sent to Dr. Tony Lee (Southern Illinois University School of Medicine-Springfield; Dept. Pharmacology) for HPLC analysis of ginsenoside content. Ginsenoside standards in the analysis included ginsenoside Rb1, Rb2, Rc, Rd, Re, Rf and Rg1. American ginseng extract contained all of the ginsenosides with identified standards in the amounts of Rb1>Rd>Re> Rc>Rg1=Rf=Rb2. The ginsenoside standards for Rg2, Rh1, Rh2 and Ro were not commercially available in the United States during these analyses and, thus, were not included as standards in the analysis of our extract. These results indicate that the ginseng extract used in our *in vivo* and *in vitro* studies contain the biologically active ginsenosides which may be responsible for the effects of ginseng extract on breast cancer cells. Indeed, as shown in Figure 1, a dose-response study examining the effects of ginseng extract on MCF-7 and MDA-MB-231 cells indicates that ginseng extract, particularly in higher doses, inhibits cell proliferation in a dose-responsive manner. The IC_{50} of ginseng extract for MCF-7 cells was 0.9×10^{-3} g/ml and for MDA-MB-231 cells was 2.1×10^{-3} g/ml. In this study, cells were plated (4×10^3) and treated with a specific dose of ginseng extract (≥12 wells/treatment group). Every two days (days 3 and 5 after plating on day 1), media was replaced with either plain media (control) or media containing ginseng extract ($5 \times 10^{-6} - 3.5 \times 10^{-3}$ g/ml). Numbers of cells/well were counted on day 6 after plating (24 hr after last treatment).

In a second study with ginseng extract, female nude mice were given different doses of ginseng extract in their drinking water (Task 1b-d). Two weeks prior to cancer cell inoculation, mice (n=12/treatment group) were given either 0.01%, 0.1% or 1 % ginseng extract in their drinking water and treatment continued throughout the experimental period. Control mice were given water only. After two weeks of treatment, mice were inoculated with 5×10^6 MCF-7 or MDA-MB-231 cancer cells in a volume of 150µl into their right flank; mice inoculated with MCF-7 cells were also implanted sc with an estradiol pellet (0.72 mg/pellet, 60-day release; Innovative Research of America). As shown in Figures 2 and 3, mice with 1% ginseng extract in drinking water exhibited a significantly decreased MCF-7 (Fig. 2) and MDA (Fig. 3) tumor size 25 and 42 days post-inoculation, respectively, when compared to water controls. The tumors in the 1% ginseng-treated mice were approximately 50% the size of the tumors in controls for the remainder of the study. The study was terminated (39 days/MCF; 63 days/MDA) when the majority of control animals began to experience morbidity as a result of tumor size. Figure 4 depicts MCF-7 tumor phenotype in nude mice upon termination of study. Upon necropsy, evidence of metastasis was present in 100% of control, MDA-inoculated mice; none of the 1% ginseng-treated animals exhibited tumors distal from the inoculation site. There were no differences in tumor size between animals on the 0.01% or 0.1% ginseng extract and water controls (data not shown). These data indicate that American ginseng extract significantly decreases MDA-MB-231 and MCF-7 cell proliferation and tumor growth and further suggest that ingestion of American ginseng may directly inhibit tumor growth.

In the final study of this Task (Task 1e,f), female Sprague-Dawley rats (n=19-20/treatment group) were given either plain drinking water (controls) or ginseng extract (0.1 or 1%) as their drinking water for 1 week prior to the administration of N-nitroso-N-methylurea (NMU; 50 mg/kg b.w.) via tail vein at 35 days of age. Our preliminary studies demonstrated that this experimental protocol produced NMU-induced tumors in 75% of control animals when NMU was administered at 35 days of age (Table 1) vs only

20% of animals when administered at 80 and 87 days of age, as we had previously proposed. Ginseng was provided *ad libitum* in the drinking water for the duration of the experiment. The results show that when comparing control animals with ginseng-treated animals there were no differences in the percentage of animals in each treatment group that presented with mammary tumors (Table 1). Furthermore, there were no significant differences between treatment groups in number of days before tumor onset after NMU injection or the size of dissected tumors at approximately 3 months after NMU injection. There were 3 control animals with very large tumors (>1000 cu. mm) that were sacrificed before the end of the study and whose tumor size was included in the calculation of results presented in Table 1. Approximately 25% of tumor-bearing rats in each treatment group presented with 2 or more mammary tumors. The data shown in Table 1 represent the averages of all tumors detected. In conclusion, results indicate that ginseng treatment did not prevent tumor formation or affect the size of tumors in NMU-treated female rats when compared to non-ginseng-exposed rats. Although we had hypothesized that ginseng would prevent NMU-induced tumors and decrease tumor size, there may be several reasons why this was not realized. NMU is a powerful carcinogen that produces mutations in mammary tissue DNA that may not be able to be reversed or attenuated by ginseng exposure. In follow-up studies, ginseng effects on the onset of mammary tumors induced by treatment with other types of carcinogens, as well as on the mammary tumors that occur in normal aging female rats could be examined. The ability of ginseng to decrease tumor size in nude mice inoculated with human breast cancer cells, but not in rat mammary adenocarcinomas, could also suggest possible species specificity in the mammary cancer response to ginseng (ie, the therapeutic efficacy of ginseng could be influenced by species differences in ginseng pharmacokinetics).

In Task 2a-b, the effects of specific ginsenosides on MDA-MB-231 and MCF-7 breast cancer cell proliferation were studied. MDA-MB-231 cells were treated on the day of plating and every 2 days with 50μM of either ginsenoside Rb1, Rb2, Rc, Rd, Re or Rf (≥12 wells/treatment group). Plates of cells were counted every 2 days. As shown in Figure 5, ginsenoside Rc produced a dramatic inhibition of cancer cell proliferation by day 2 of treatment. Cell proliferation was decreased to approximately 25% of control after 8 days of treatment. Interestingly, none of the other ginsenosides affected MDA-MB-231 cell proliferation at any time after treatment. A second ginsenoside, Rh2, has recently been identified as a constituent of American ginseng (Yuan et al., 2001) that also inhibits cancer cell proliferation (Oh et al., 1999), including the inhibition of MCF-7 cell proliferation. In the next study, MCF-7 cells were plated and treated 24hr later with ginsenoside Rc (Sigma Chemical Co., St. Louis, MO) or ginsenoside Rh2 (Sequoia Research Products, Oxford), in doses of 1-50μM, every 2 days for 6 days. Ginsenoside doses of 10-50μM produced dramatic decreases in cell proliferation (Figure 6). Whereas doses of 25-50μM of ginsenoside Rc produced a maximal 50% inhibition of cell proliferation, the same doses of ginsenoside Rh2 produced a 90-100% inhibition relative to vehicle controls. These results suggest that treatment of MCF-7 cells with ginsenoside Rc produces cytostatic effects, whereas ginsenoside Rh2 is primarily cytotoxic. Combining the IC50 dose of ginsenoside Rh2 (18μM) with ginsenoside Rc (IC50=21μM) dramatically enhanced the efficacy of ginsenoside Rc on inhibition of cancer cell proliferation (IC50=1.9μM).

In Task 3, the effects of specific ginsenosides in vivo on tumor initiation and growth in nude mice inoculated with human breast cancer cells and in NMU-treated female rats were proposed. When this project was initiated in year 2 of grant, it was discovered that ginsenoside Rc was temporarily unavailable from Sigma Chem. Co. Ginsenoside Rc was obtained from other sources, including Indofine and Research Plus. However, the Rc from these sources was not bioactive in our in vitro proliferation assays. Mass spectrometry and NMR analyses of the 3 different ginsenoside Rc drugs were performed and the results some difference in enantiomerism but revealed little other difference between the 3 compounds. In the meantime, Rh2 had become commercially available from Sequoia Research Products in Oxford, UK. Very recently, it was shown that American ginseng contained ginsenoside Rh2 (Yuan et al., 2001), as well as Rc, and that ginsenoside Rh2 had anti-proliferation activity in cancer cell proliferation assays (Oh et al., 1999; [3]see results in Task 2) Thus, current studies are being conducted in nude mice inoculated with MCF-7 cells and treated with Rh2 (5μM) in their drinking water. After 11 months of unavailability, Sigma ginsenoside Rc is again available. Studies will be conducted to examine the effects of ginsenosides Rc and Rh2, alone and in combination, on MCF-7 and MDA-MB-231 human breast cancer cell proliferation in nude mice in vivo. A no-cost extension has been requested to finish these studies.

Key Research Accomplishments

- American ginseng extract inhibited MCF-7 and MDA-MB-231 human breast cancer cell proliferation *in vitro* in a dose-dependent manner.
- A 1% American ginseng extract in drinking water decreased tumor size by ≥50% in female nude mice inoculated with either MCF-7 or MDA-MB-231 human breast cancer cells.
- American ginseng extract contains 7 identifiable ginsenosides, as determined by HPLC, which may be responsible for the anti-proliferating effect of the ginseng extract on human breast cancer cells *in vitro* and *in vivo*.
- American ginseng extract did not affect NMU-induced tumor onset or tumor size in female Sprague-Dawley rats
- Ginsenosides Rc and Rh2 significantly decreased MCF-7 cell proliferation *in vitro*, and a combination dose of Rc and Rh2 was more potent in inhibiting cell proliferation than the effect of either ginsenoside alone.
- Whereas ginsenoside Rc appeared to have cytostatic effects on cell proliferation, ginsenoside was cytotoxic, suggesting different mechanisms of ginsenoside action on MCF-7 cells.

Reportable Outcomes

Abstract:

Rice, J.A., Compardo, M.T., Scolari, K.A., and Murphy, L.L. Ginsenoside Rc inhibits human breast cancer cell proliferation *in vitro*. Molecular Biology of the Cell (suppl) 11: 452, 2000.

Murphy, L.L., Rice, J.A., Zong, W. Ginsenosides Rc and Rh2 inhibit MCF-7 cell proliferation through distinctly different mechanisms. Molecular Biology of the Cell (suppl) 12:764, 2001.

Invited Presentation:

Murphy, L.L. American ginseng and its effects on human breast and prostate cancers *(Invited oral presentation at the Wisconsin Ginseng Growers Association in Wausau, WI, 2000)*

Murphy, L.L., Lam, F. Treatment of cancer with Chinese herbals is enhanced by co-administration with American ginseng. *(Invited oral presenation at the First International Conference of the Modernization of Chinese Medicine, Hong Kong, 2002)* – this work was supported in part by American WildSenergy Inc.

Murphy, L.L. American ginseng: A new alternative in breast cancer therapeutics? *(Invited oral presentation at the Cancer 2002: Advances in Research, Prevention, Diagnosis and Treatment conference, Springfield, IL, 2002)*

Invited Presentation w/Abstract:

Murphy, L.L. American ginseng and its effects on human breast and prostate cancers. *(Invited oral presentation at the International Ginseng Conference, Leeds, NY, 2000)*

Murphy, L.L., Zong, W., Rice, J.A. Effects of American ginseng and Chinese herbal powder, alone and in combination, on human breast cancer MCF-7 cells *in vitro*. *(Invited oral presentation at the New York 21st Century Chinese Medicine Forum, New York City, 2001)* - this work was supported in part by American WildSenergy Inc.

Murphy, L.L., Rice, J.A. , Compardo, M.T. American ginseng inhibits tumor growth in athymic nude mice inoculated with human breast cancer MCF-7 or MDA-MB-231 cells. *(Invited oral presentation at the International Scientific Conference on Complementary, Alternative and Integrative Medicine Research, Boston, MA, 2002)*

Manuscript:

Rice, J.A. and Murphy, L.L. Ginsenoside Rc, a constituent of American ginseng extract, inhibits breast cancer cell proliferation in vitro. *(In preparation for submission to Cancer Letters, 2002)*

Conclusions

The most notable findings of this research project include our findings that American ginseng extract decreases human breast cancer growth in both in vitro and in vivo models. Moreover, components of American ginseng, ginsenoside Rc and Rh2, inhibit breast cancer cell proliferation *in vitro*. These findings are the first to suggest that these ginsenosides may be responsible for the anti-proliferating actions of ginseng extract on human breast cancer cell proliferation. Preliminary studies indicate that American ginseng extract and ginsenoside Rc may inhibit breast cancer cell cycle progression, whereas ginsenoside Rh2 may be cytotoxic to breast cancer cells. Thus, different ginsenosides may have very different mechanisms of action. Future studies would examine the cell cycle regulatory genes/proteins that mediate the actions of ginsenosides on cancer cell proliferation. The nude mouse findings would also suggest that ginseng and its ginsenosides may decrease angiogenesis and prevent or slow metastasis of the breast cancer tumor. In future studies, the mechanisms by which ginseng/ginsenosides may alter these physiological processes of tumor growth and differentiation would be examined. The inability of ginseng to prevent NMU-induced mammary tumor initiation and growth indicates a potential limitation to ginseng as a preventative against certain mutagenic carcinogens. However, these studies were overall supportive of American ginseng and its ginsenoside constituents as potential preventative or therapeutic agents for breast cancer.

References

Oh, M., Choi, Y.H., Choi, S-H., Chung, H-Y., Kim, K-W., Kim, S.I., and Kim, N.D. Anti-proliferating effects of ginsenoside Rh2 on MCF-7 human breast cancer cells. International Journal of Oncology 14: 869-875, 1999.

Yuan, C.-S., Wang, X., Wu, J.A., Attele, A.S., Xie, J.-T., and Gu, M. Effects of Panax quinquefolius on brainstem neuronal activities: Comparison between Wisconsin-cultivated and Illinois-cultivated roots. Phytomedicine 8: 178-183, 2001.

Appendices

Please see Figures 1-6 and Table 1 on the following pages.

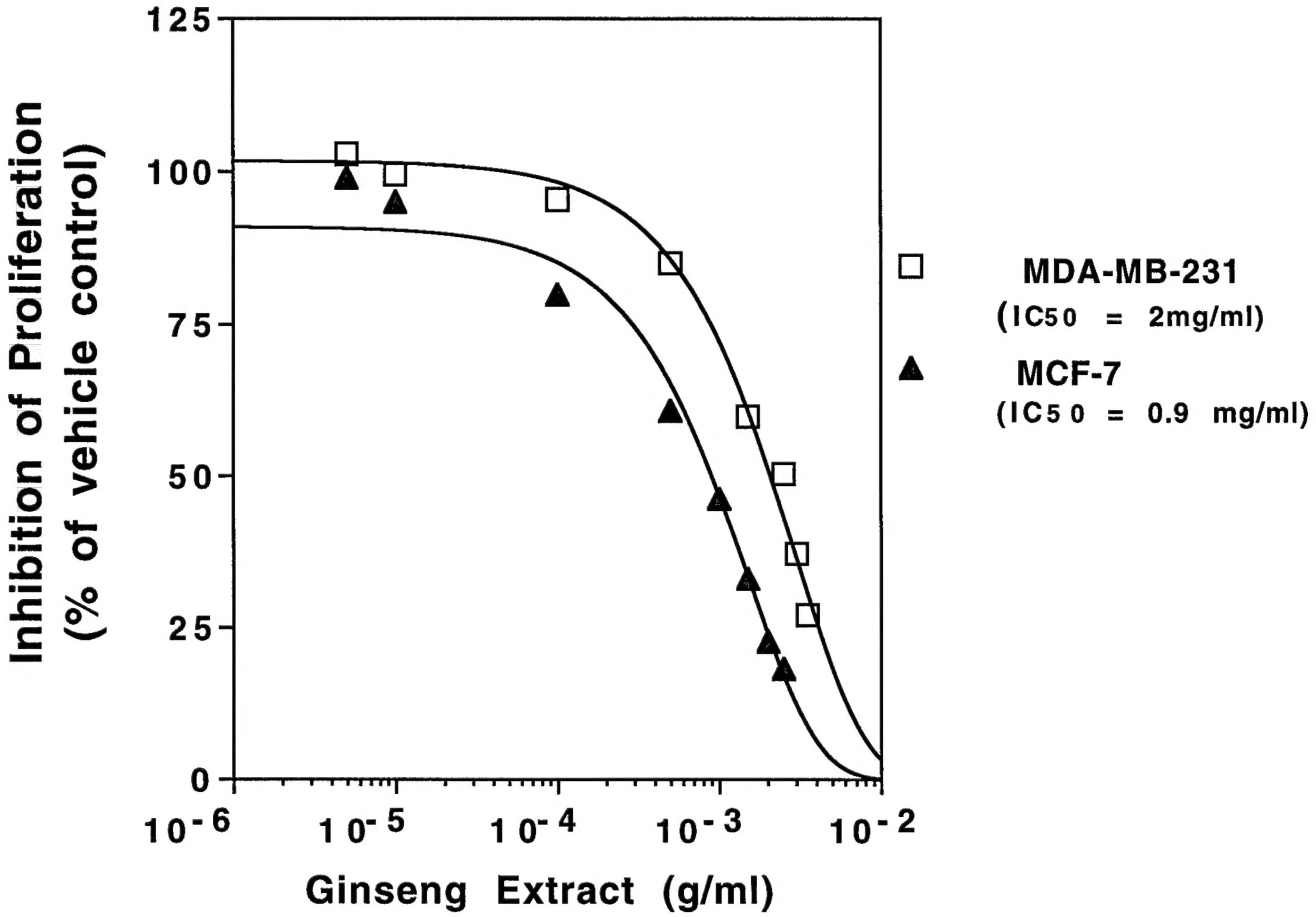

Figure 1 Effect of ginseng extract (GE) on proliferation of MCF-7 and MDA-MB-231 breast cancer cells in culture. Cells were treated with specific doses of GE every 2 days and were counted after 6 days in culture (24 hr after last treatment). Data are graphed as percent of cells relative to vehicle control.

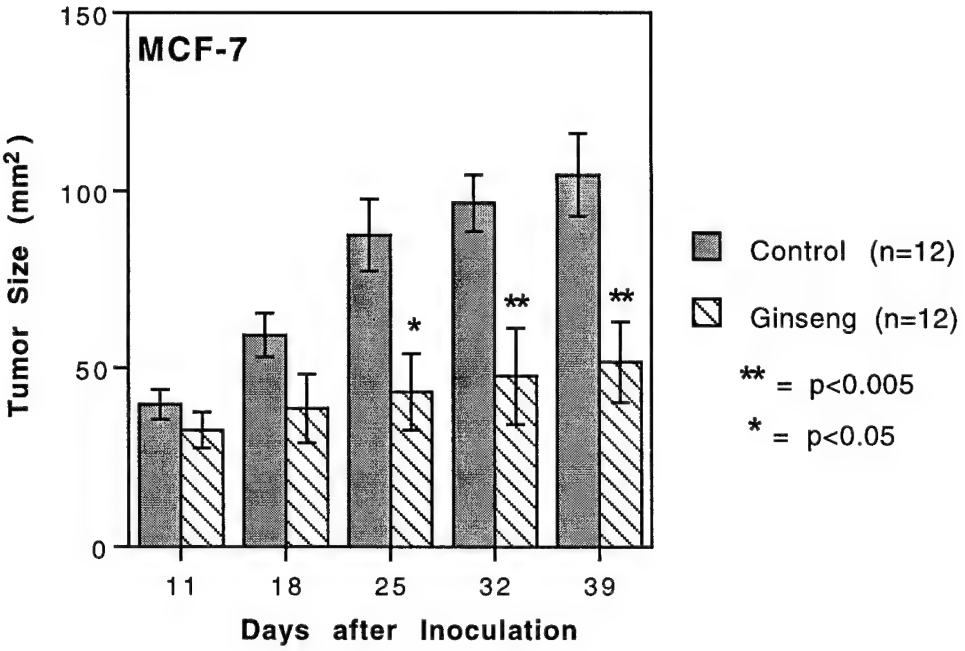

Figure 2 Effect of a 1% ginseng extract on tumor growth in female nude mice inoculated with MCF-7 breast cancer cells. Drinking water containing 1% ginseng extract was provided *ad libitum* to mice two weeks prior to inoculation with 5 x 10^6 MCF-7 cells. Mice were maintained on the 1% GE throughout the experimental period. Tumor size was measured weekly.

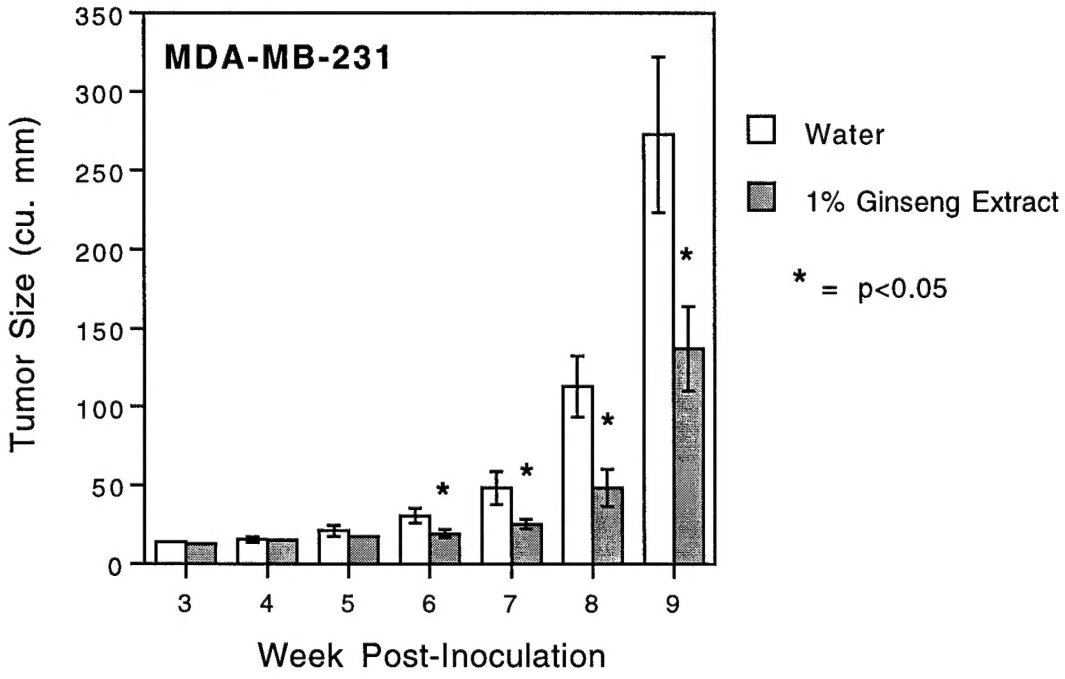

Figure 3 Effect of a 1% ginseng extract on tumor growth in female nude mice inoculated with MDA-MB-231 breast cancer cells. Drinking water containing 1% American ginseng extract was provided to mice two weeks prior to inoculation with 5 x 10^6 MDA-MB-231 cells. Mice were maintained on the ginseng extract throughout the experimental period. Tumor size was measured weekly. * = $p \leq 0.05$ relative to water control

Figure 4 Representative nude mice from a control group (upper two mice) or 1% ginseng extract-treated group (bottom two mice). Note signs of necrosis in control mice. An estrogen pellet can be seen on the second mouse from the top.

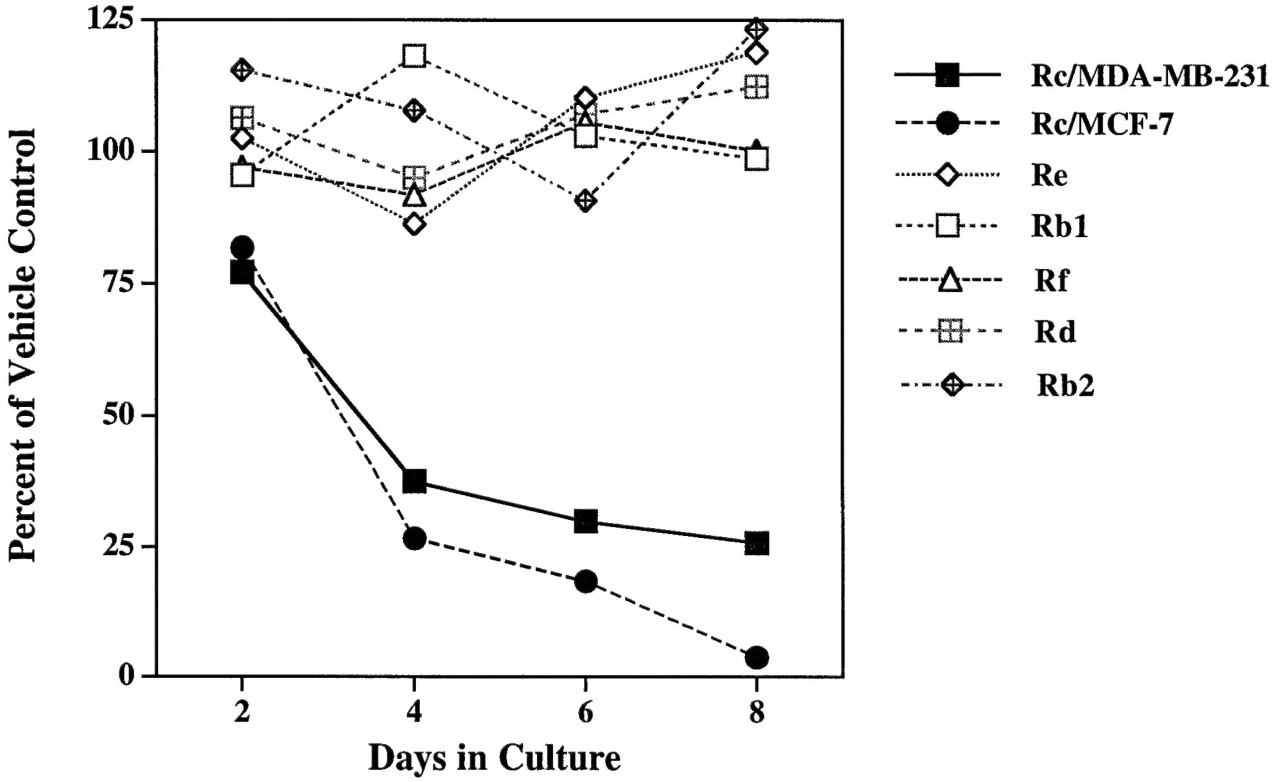

Figure 5 Effect of different ginsenosides on proliferation of MCF-7 and MDA-MB-231 cells in culture. MDA cells were treated with a 50μM dose of ginsenosides every 2 days; both MDA and MCF-7 cells were treated with a 50μM dose of ginsenoside Rc. Cells were counted on Day 8 of treatment and data were graphed as a percent of vehicle control.

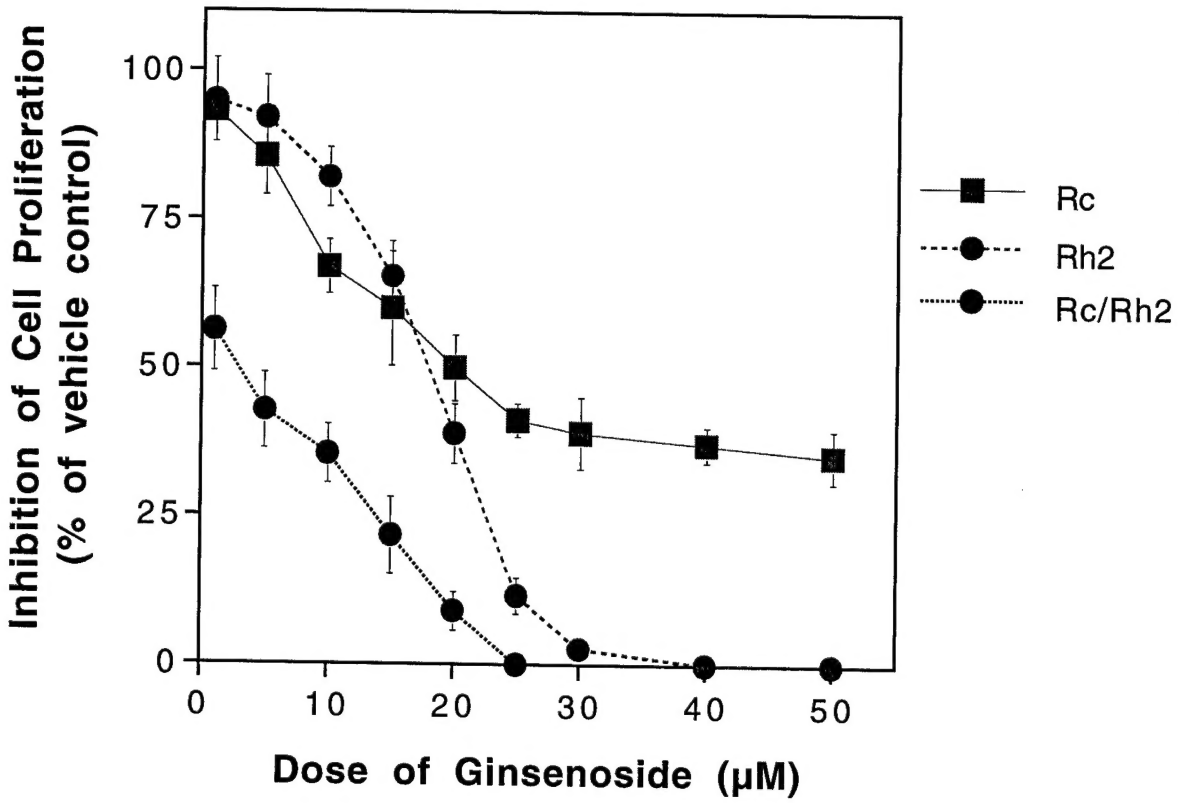

Figure 6 Effect of ginsenosides Rc and Rh2 on proliferation of MCF-7 breast cancer cells in culture. Cells were treated with a wide dose range of either ginsenoside Rc or Rh2, or a combination of Rh2 (18μM) and Rc, every 2 days. Cells were counted on Day 6 of treatment and data were graphed as a percent of vehicle control.